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Gastrin Exerts Pleiotropic Effects on Human Melanoma Cell Biology[1]

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Abstract

The effects of gastrin (G17) on the growth and migration factors of four human melanoma cell lines (HT-144, C32, G-361, and SKMEL-28) were investigated. The expression patterns of cholecystokinin $(CCK)_A$, CCK_B, and CCK_C gastrin receptors were investigated in these cells and in seven clinical samples by means of reverse transcription polymerase chain reaction. Melanoma cells appear to express mRNA for CCK_C receptors, but not for CCK_A or CCK_B receptors. Although gastrin does not significantly modify the growth characteristics of the cell lines under study, it significantly modifies their cell migration characteristics. These modifications occur at adhesion level by modifying the expression levels of α_v and β_3 integrins, at motility level by modifying the organization of the actin cytoskeleton, and at invasion level by modifying the expression levels of matrix metalloproteinase 14. We recently demonstrated the presence of CCK_B receptors in mouse endothelial cells involved in glioblastoma neoangiogenesis. Chronic *in vivo* administration of a selective CCK_B receptor antagonist to mice bearing xenografts of human C32 melanoma cells significantly decreased levels of neoangiogenesis, resulting in considerable delays in the growth of these C32 xenografts. In conclusion, our study identifies the pleiotropic effects of gastrin on melanoma cell biology.

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Introduction

In the case of metastatic melanomas, the rate of complete and durable response remissions merely ranges from 5% to 20% and can be achieved with biochemotherapy, a process that includes the use of biomolecules [interleukin (IL)-2 and interferon-α] in combination with various cytotoxic agents (dacarbazine, cisplatin, and vinblastine) [1,2]. Insulin-like growth factors (IGFs) are among those biomolecules that play various major roles in the biology of many cancer types in general [3], and of melanomas in particular [4,5]. We hypothesize that gastrin (G17) could also influence the biologic behavior of human melanomas for the following reasons. Reubi et al. [6] analyzed 406 human tumors of various origins and observed that medullary thyroid carcinomas, small cell lung cancers, and astrocytomas (three types of cancers with "neural" origins) frequently express gastrin receptors [i.e., the cholecystokinin $(CCK)_B$ receptor described below], whereas their pure epithelial counterparts—differentiated thyroid cancers of the follicular and papillary types and non–small cell lung cancers—do not. Melanomas are also reported to have a neural origin. Melanomas could thus express gastrin receptors. As far as we know, neither Reubi et al. nor any other group has investigated whether melanomas express gastrin receptors. As recently reviewed by Rozengurt and Walsh [7] and Dockray et al. [8] and as summarized by Lefranc et al. [9], three receptors to which gastrin can bind have already been cloned. The CCK_A (also named CCK_1) receptor is highly selective (from 500- to 1000-fold) toward the sulfated analogues of CCK, and especially of CCK-8 [7,8,10]. The CCK_B receptor (also called CCK_2 and the gastrin receptor) is less selective than CCK_A because it does not require a sulfated tyrosine residue of CCK-8 [7,8,10]. Gastrin has also been reported to bind to the 78-kDa gastrin-binding protein (GBP; i.e., the so-called "CCK_C gastrin receptor") [11]. Several GBPs have been described [12–16] in addition to the three cloned receptors. Although human glioblastoma cells do not display CCK_A and CCK_B receptors [6,12], we demonstrated that gastrin significantly modifies cell proliferation [17–19] and migration of human glioblastoma cells [12,18,20,21]. In fact, we evidenced that the biologic gastrin-mediated effects on these glioblastoma cells are mediated by binding sites for the C-terminal heptapeptide of gastrin (G17) [12]. We also emphasize that gastrin modulates proliferation and apoptosis levels in a large variety of cancers originating from the gastrointestinal tract (*cf.* Refs. [7,10] for reviews; also Refs. [22,23]). In addition to its roles in cell proliferation,

Abbreviations: CCK, cholecystokinin; G17, gastrin; IGF, insulin-like growth factor; IL-8, interleukin-8; MMP, matrix metalloproteinase; MRDO, maximum relative distance to the origin; MTT, (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; PI3-K, phosphoinositide 3-kinase

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apoptosis, and migration of a large variety of cancer cells, gastrin has potent proangiogenic effects, as we recently demonstrated in the case of experimental glioblastomas [9].

The primary objective of this study was to characterize the presence or absence of mRNA for the CCK_A, CCK_B, and CCK_C receptors. This was done by means of reverse transcription polymerase chain reaction (RT-PCR) techniques (because no reliable antibodies exist for protein analysis) on four human melanoma cell lines (the HT-144, C32, G-361, and SKMEL-28 models) and seven human melanoma metastases (used as a control). We then characterized *in vitro* the influence of gastrin on global growth [(3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay], cell proliferation (flow cytometry), apoptosis (flow cytometry), and migration by means of a separate analysis of cell motility (quantitative videomicroscopy) and cell invasion processes (Matrigel-coated Boyden chambers) in the abovementioned human melanoma cell lines. The patterns of matrix metalloproteinase (MMP) and integrin mRNA expression, determined by means of cDNA microarrays, led us to analyze the influence of gastrin on the MMP-14 and $\alpha_v\beta_3$ integrin levels of expression by means of Western blot analysis and quantitative immunofluorescence. Using a scratch wound assay, we also investigated whether gastrin significantly modified the cytotoxic effects induced by cisplatin and dacarbazine on human HT-144 melanoma cells. As we had recently demonstrated the involvement of the CCK_B receptor in neoangiogenesis in experimental glioblastomas [9], we also investigated in this study whether a specific CCK_B gastrin receptor antagonist displayed antiangiogenic effects in human C32 xenografts grafted subcutaneously onto nude mice and subsequently influenced the growth rates of these xenografts.

Materials and Methods

Cell Lines and Culture Media

The four human melanoma cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and were maintained *in vitro* in accordance with the recommendations of the ATCC. The C32 cells were cultured in minimal essential medium (MEM; GibcoBRL/Life Technologies/Invitrogen, Merelbeke, Belgium), to which 5% fetal calf serum (FCS; GibcoBRL) was added. SKMEL-28 cells were cultured in RPMI medium (GibcoBRL), to which 10% FCS was added. HT-144 cells were cultured in MEM with 10% FCS, with the addition of essential amino acids. The G-361 cells were cultured in OPTIMEM medium (GibcoBRL), to which 5% FCS was added.

All the media were supplemented with a mixture of 0.6 mg/ml glutamine (GibcoBRL), 200 IU/ml penicillin (GibcoBRL), 200 IU/ml streptomycin (GibcoBRL), and 0.1 mg/ml gentamicin (GibcoBRL). The FCS was heat-inactivated for 1 hour at 56°C.

Human MCF-7 breast cancer cells (ATCC code HTB-22) were used as a control for p53-induced expression.

Clinical Samples

We analyzed the pattern of gastrin receptor expression in seven metastases from human melanoma patients who had undergone surgery at the Erasmus University Hospital. The seven clinical samples under study were obtained from the Tumor Tissue Collection at the Laboratory of Pathology, Erasmus University Hospital.

Compounds

Gastrin (pGlu–Gly–Pro–Trp–Leu–Glu–Glu–Glu–Glu–Glu–Ala–Tyr–Gly–Trp–Met–Asp–Phe–NH_2), cisplatin, and dacarbazine were purchased from Sigma (Bornem, Belgium). The L365,260 (3*R*-3-[*N* -(3-methylphenyl)ureido]-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepine-2-one) compound was provided by ML Laboratories PLC (London, UK).

RT-PCR Analyses to Determine the CCK_A, CCK_B, and CCK_C Receptor mRNA

The procedure used here is identical to the one we recently described in detail [9]. All the PCR products were sequenced by GenoScreen (Lille, France) by means of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

As indicated in Table 1, four primers (p1–p4) were assayed for the CCK_A, two (p5 and p6) for the CCK_B, and two (p7 and

Table 1. Description of the Forward and Reverse Primers Used in the Present Study.

Targeted Gene (Human)	Forward Primers	Reverse Primers	Location	Positions	PCR Product Sizes
β-Actin	5′ -AAATCGTGCGTGACATTAAGG-3′	5′ -CTAAGTCATAGTCCGCCTAG-3′	Interexons	661–1185	525
CCK_A receptor					
p1	5′ -GTTGACAGCCTTCTTGTG-3′	5′ -CAGATTAAAGGTAGATACACTCAC-3′	Interexons	163–549	387
p2	5′ -TGCAAACCCTTACAGTCCC-3′	5′ -CCACCATCATCACACTTCC-3′	Interexons	583–835	253
p3	5′ -CAACTTGGTGCCTTTTACC-3′	5′ -TCCATATGCCACCATCATC-3′	Interexons	693–843	151
p4	5′ -CTCGTACAGCCATATGAGTG-3′	5′ -GAACTGCCTAGAAACCAATC-3′	Noncoding	1401–1651	251
CCK_B receptor					
p5	5′ -TCATTCACTTGCTGAGCTAC-3′	5′ -CAGTGTCATGTTTCTATGGG-3′	Partly coding	1314–1774	461
p6	5′ -CCCATAGAAACATGACACTG-3′	5′ -GAGATTAGGCCTGATGGTATT-3′	Noncoding	1755–1988	234
CCK_C receptor					
p7	5′ -TATCACGACCGAGAAAACTT-3′	5′ -CGCTACATCCACACCAACTT -3′	Interexons	1559–1814	256
p8	5′ -TGGATAGTATTTTAGCGAGTCTG-3′	5′ -CTTCTCGTTACTCTGATAAATCTAG-3′	Interexons	1981–2418	438

p8) for the CCK_C receptors. The exact locations targeted by each of these primers with respect to the mRNA for the CCK_A, CCK_B, and CCK_C receptors are illustrated in Table 1.

The thermal profiles were as follows: predenaturation for 4 minutes at 95°C; denaturation for 45 seconds at 95°C; annealing for 45 seconds at 57°C (p1, p3, and p4), 58°C (p2, p6, p7, and p8), and 62°C (β-actin, p5); elongation for 1 minute and 15 seconds at 72°C; and a final elongation for 10 minutes at 72°C. The PCR products were obtained after 40 thermal cycles, with the exception of the β-actin, which was obtained after 30 thermal cycles.

cDNA Microarray to Determine the mRNA Patterns of Expression for Metalloproteinases and Integrins in Human Melanoma Cells

The method that we used to check the quality of the extracted RNA before submitting the four human melanoma cell lines under study to the metalloproteinase- and integrin-related cDNA microarray approach and to this cDNA microarray approach is described in detail elsewhere [9].

We used two types of microarrays provided by Superarray (Bethesda, MD), referred to as the "GEArray Original Series Human Integrin Gene Array" and the "GEArray Original Series Human Metalloproteinase Gene Array." These arrays are designed to assess the expression levels of 15 α-integrin and 8 β-integrin subunits, in addition to 19 metalloproteinases and 3 metalloproteinase tissue inhibitors (TIMP-1, TIMP-2, and TIMP-3) spotted in duplicate. The array kits were used, in accordance with the procedures recommended by the manufacturer, to assess the levels of gene expression in equal amounts of total RNA extracted from human HT-144 melanoma cells (*cf.* Figures 2*B* and 3*C*). The hybridization results were visualized by a Fuji-BAS5000 scanner, and the relative amounts of mRNA transcripts were quantified using an AIDA image analyzer software (Raytest Benelux, Tilburg, The Netherlands).

Western Blot Analyses [MMP-14, p53, PTEN, poly(ADP-ribose) polymerase (PARP), Akt, phospho-Akt, and mTOR]

Cell extracts were prepared from lyses of subconfluent cells in ice-cold phosphate-buffered saline (PBS) containing a MiniComplete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Twenty micrograms of proteins (evaluated by the BCA protein assay; Pierce/Perbio Science, Erembodegem, Belgium) was loaded onto a denaturating polyacrylamide gel (10%) and blotted onto a Polyscreen*PVDF membrane (NEN Life Science Products, Boston, MA). The Akt (dilution 1/1000), $^{Ser\ 473}$p-Akt (dilution 1/1000), $^{Thr\ 308}$p-Akt (dilution 1/500), mTOR (dilution 1/1000), and PTEN (dilution 1/750) antibodies were purchased from Cell Signaling (Beverly, MA); MMP-14 antibody (dilution 1/50) was from Oncogene Research Products (Calbiochem/VWR, Leuven, Belgium); p53 antibody (dilution 1/1000) was from Calbiochem; and rat tubulin (dilution 1/2000) was from Abcam (Cambridge, UK). The secondary antibodies were purchased from Pierce, with the exception of the rabbit anti-rat antibody, which was supplied by Abcam.

The integrity and quantity of the extracts were determined by means of tubulin immunoblotting. Blots were developed using the Pierce Supersignal Chemiluminescence system (Perbio Science).

Determination of Protein Expression by Quantitative Fluorescence

Fluorescent detection of protein expression in cells cultured on glass coverslips was performed as previously described [9,19,21,24]. The specific binding of the primary antibodies was detected by Alexa Fluor 488– or Alexa Fluor 594–conjugated secondary antibodies (10 μg/ml; Molecular Probes, Inc., Eugene, OR). Fluorescent phallacidin conjugated to Alexa Fluor 488 fluorochrome (Molecular Probes, Inc.) was selected to label the fibrillar actin, and Alexa Fluor 594–conjugated DNAseI (Molecular Probes, Inc.) was chosen to stain the globular actin to conduct an investigation into the organization of actin cytoskeleton. α_v and β_3 integrin antibodies were provided by BD Transduction Laboratories (Erembodegem, Belgium) and were used at 1/100 dilution. The staining protocols are described elsewhere [9,19,21,24].

The fluorescence intensities of the fluorochromes were quantified by a computer-assisted fluorescent Olympus AX70 microscope (Omnilabo, Antwerp, Belgium), equipped with a Megaview2 digital camera and an analySIS software (Soft Imaging System, Munster, Germany), as described in detail elsewhere [9,19,21,24]. All the cells for analysis were selected using the computer mouse to draw contours on the video screen (connected to the computer-assisted microscope) based on a phase-contrast image. One hundred cells were quantified for each experimental condition.

In Vitro *Overall Growth Determination*

The four human melanoma cell lines were incubated in 96-microwell plates for 24 hours (at a concentration of 40,000 cells/ml culture medium) to ensure adequate plating prior to the determination of cell growth. The influence of gastrin on overall growth rates of the four human melanoma cell lines under study was determined by means of the colorimetric MTT assay, as previously described [17,19]. Each experiment was conducted in sextuplicate. Nine concentrations ranging from 10^{-12} to 10^{-8} M (with a semilog decrease in concentration) were assayed for gastrin.

Flow Cytometry Analyses to Determine Apoptotic Versus Nonapoptotic-Related Cell Death in Human HT-144 and C32 Melanoma Cells

Flow cytometry analyses of apoptotic-related *versus* nonapoptotic-related HT-144 and C32 cell death were performed in accordance with the experimental protocol described in detail by Darzynkiewicz et al. [25]. In summary, after various treatments with gastrin and cytotoxic drugs [such as cisplatin (*cf.* Figure 4*E* and its legend) and dacarbazin; data not shown], the cells were incubated in darkness in an Annexin V–FITC (V) and propidium iodide (PI) solution for 15 minutes at 4°C (Annexin V–FITC Kit; Immunotech, Marseille, France). Data from around 10,000 cells were recorded on a logarithmic scale for each sample. An "XL System II"

(Beckman Coulter, Miami, FL) was used to give a precise definition of both the percentage of cells in the apoptotic and/or nonapoptotic compartments and the percentage of normal cells (i.e., those that apparently remained unaffected by the various treatments). The so-called normal cells were negative for both the Annexin V and PI staining, whereas the apoptotic cells were positive for Annexin V but negative for PI. The nonapoptotic cells (whose cell membranes have holes) were positive for both PI and Annexin V. Each experiment was conducted in triplicate.

The cell cycle kinetics of the HT-144 and C32 melanoma cells treated with gastrin or left untreated was determined by flow cytometry analyses of the PI nuclear staining, as described in detail by de Launoit et al. [26].

In Vitro *Invasion Assay*

The Boyden transwell invasion system (BD BioCoat Matrigel invasion chambers; BD Biosciences Discovery Labware, Bedford, MA) was used to evaluate cell invasiveness. These invasion chambers were divided into two compartments separated by an 8-μm pored polyethylene membrane coated with Matrigel (10 μm thickness). After rehydration of the inserts, between 40,000 and 60,000 cells (depending on the melanoma cell type) in 500 μl of serum-supplemented medium were seeded in the upper chambers, whereas the lower chambers were filled with 500 μl of serum-supplemented medium. G17 was added to the upper compartment at concentrations ranging from 10^{-12} to 10^{-8} M immediately after the seeding process. The cells were allowed to invade through the matrix at 37°C for 24 hours. The noninvading cells remaining on the upper surface of the coated membrane were removed with a cotton-tipped swab. The invading cells on the lower surface of the membrane were fixed, stained with Diff-Quick (Merz+Dade AG, Baxter, Belgium), and counted with an inverted-phase contrast microscope.

To further normalize the data in accordance with cell growth, the experimental conditions of each Boyden chamber were reproduced simultaneously in a 24-well plate coated with Matrigel. The invasive rate was obtained by reporting the number of invading cells in each Boyden chamber compared with the total number of cells in the corresponding well, computed with a cell counter (Coulter-Particle-Counter-Z2; Analys, Ghent, Belgium) after trypsin treatment. Each experiment was conducted in triplicate.

In Vitro *Motility Assay*

Human melanoma cell motility levels were characterized using a device (described elsewhere) [18,21,27] that enables the quantification of the trajectories of living cells maintained in culture. The greatest linear distance migrated by each cell was calculated based on these trajectories. This distance is, in fact, the maximum relative distance from the point of origin [i.e., the maximum relative distance to the origin (MRDO) quantitative variable] [18,21,27]. All the experiments were performed over 8 hours, and one image was recorded every 4 minutes. Because the analyses were conducted in triplicate, a minimum of 82 cells and a maximum of 170 cells were analyzed in each experimental condition. Analyses in triplicate imply that each experiment was conducted independently three times. The effect of gastrin on human melanoma cell motility levels was analyzed from 10^{-12} to 10^{-8} M.

In Vitro *Scratch Wound Assay*

The HT-144 melanoma cells were grown to confluence in six-well dishes. The cells were then pretreated with 10 nM gastrin for 1, 3, 7, or 24 hours in a serum-restricted medium (5%). Scratch wounds were made by creating a linear denuded region using a pipette tip (as illustrated in Figure 4, *A* and *B* and their legends). The cells were washed twice with PBS prior to their incubation with the cytotoxic agent (cisplatin or dacarbazine) at 0.1, 1, and 10 μM in a serum-restricted medium for 62 additional hours of postgastrin treatment. Four fields per well were photographed twice daily until wound healing was observed in the control. A software developed in our laboratory was used to quantify the area filled by the cells (within the frame illustrated in Figure 4, *A* and *B*) over the period of the experiment.

Characterization of the Effects Induced In Vivo *by a Gastrin Antagonist on the Growth and Level of Neoangiogenesis in Human C32 Melanoma Cells Xenografted Subcutaneously onto the Flanks of Nude Mice*

The effects induced *in vivo* by gastrin receptor antagonist L365,260 were determined in neoangiogenesis in subcutaneous human C32 melanoma xenografts by administering L365,260 (10 mg/kg, i.p.) five times a week for eight consecutive weeks. Treatments started on the 14th day posttumor graft.

Subcutaneous C32 xenografts were performed by grafting 2×10^6 C32 human melanoma cells onto the left flanks of 8-week-old female nu/nu mice (weight 21–23 g; BioServices, Uden, The Netherlands). We chose the C32 model from the four human melanoma models under study due to the highly reproducible tumor developments (100%) obtained for this model; no such success rates were ever obtained for the remaining three models (data not shown).

Tumor size was measured twice weekly by means of a caliper and expressed as an area (square millimeters) by multiplying the two largest perpendicular diameters. We were thus able to evaluate the potential antiangiogenic L365,260-induced effects on C32 tumor growth rates. Because the average tumor size had attained 500 mm^2 in the control group, the endpoint of the C32-related *in vivo* experiments was the sacrifice, for ethical reasons (in a CO_2 atmosphere), of all the nude mice bearing subcutaneous xenografts on the 85th day posttumor graft. The tumors were removed, fixed in buffered formalin for 5 days, embedded in paraffin, and cut into 5-μm-thick sections; histologic slides were stained with hematoxylin and eosin for vessel counts (*cf.* Figure 5*B*). We also investigated whether the L365,260 treatment modified C32 tumor cell heterogeneity or proliferation. We used an anti-p53 antibody (Dakocytomation, Glostrup, Denmark) to analyze the effects of L365,260 on tumor cell heterogeneity and the MIB-1 antibody (Dakocytomation), which targets the Ki-67–related cell proliferation marker, to analyze cell proliferation levels.

All the *in vivo* experiments described in the current study were performed based on authorization no. LA1230509 of the Animal Ethics Committee of the Federal Department of Health, Nutritional Safety, and the Environment (Belgium).

Characterization of the Patterns of Immunohistochemical Expression of S100B and HMB-45 to Prove That the Human Melanoma Cell Lines Used Here Exhibited Actual Characteristics of Human Melanoma

We used anti-S100B and anti-HMB45 antibodies (Biogenex, San Ramon, CA) to investigate whether the C32 and HT-144 human melanoma models used in this study still exhibited the actual biologic characteristics of human melanomas. The immunohistochemical procedures were identical to those previously described in detail [28].

Statistical Analyses

Statistical comparisons were made by conducting the Kruskal-Wallis test (a nonparametric one-way analysis of variance). Where this test revealed some significant differences, we compared pairs of groups by applying the Mann–Whitney *U* test. All the statistical analyses were performed using Statistica (Statsoft, Tulsa, OK).

Results

Characterization of mRNA Expressions for the CCK_A, CCK_B, and CCK_C Receptors in Four Human Melanoma Cell Lines and Seven Clinical Samples

Figure 1 illustrates the mRNA expression pattern of the CCK_A (Figure 1, *A* and *B*), CCK_B (Figure 1, *C* and *D*), and CCK_C (Figure 1, *E* and *F*) receptors in four human melanoma cell lines (*lanes 4–7* in Figure 1, *A*, *C*, and *E*) and seven human melanoma metastases (*lanes 8–14* in Figure 1, *B*, *D*, and *F*) assessed by means of RT-PCR. The four cell lines include the HT-144, G-361, C32, and SKMEL-28 models. An RT-PCR for β-actin was performed using all the extracts as quality control (Figure 1, *G* and *H*).

Figure 1, *A* and *B* shows that in the four melanoma cell lines and the seven human melanoma metastases, primer p2 (*cf.* Table 1) failed to evidence any mRNA for CCK_A. Lane 3 in Figure 1, *A* and *B* represents the positive control in the shape of a specimen of gallbladder tissue. Primer p1 (*cf.* Table 1) also failed to evidence any mRNA for CCK_A in human melanoma cell lines and clinical samples of melanoma metastases (Table 2), whereas primers p3 and p4 (Table 1) revealed the presence of parts of the CCK_A receptor mRNA in these biologic samples (Table 2).

Figure 1, *C* and *D* shows that CCK_B receptor mRNA can be evidenced in human melanoma cell lines as well as in clinical samples of human melanoma metastases; however, these data were obtained with primer p6, which was located in a noncoding region of the CCK_B receptor (*cf.* Table 1). In contrast, primer p5 evidenced CCK_B receptor mRNA in only one of the seven melanoma metastases and in none of the four human melanoma cell lines (Table 2).

Thus, because two or three of the four primers used here to target the CCK_A receptor mRNA failed to demonstrate the presence of any mRNA, the present data strongly suggest that functional CCK_A receptors were neither present in the four human melanoma cell lines under study nor in the seven clinical samples of human melanoma metastases. The same conclusion may be drawn for the CCK_B receptor. These conclusions are supported by functional studies because we observed no significant binding of radioiodinated CCK or gastrin in any of the four human melanoma cell lines under study (data not shown). These functional studies were conducted by means of radioactive binding assays, as described in detail elsewhere [12]. We did not succeed in demonstrating either the presence or absence of the CCK_A and CCK_B receptors at protein level in the four human melanoma cells lines because the only commercially available antibodies against the CCK_A and CCK_B receptors failed to reveal the presence of any CCK_A receptors in the human gallbladder tissue (reference tissue; data not shown) and of any CCK_B receptors in the human brain tissue (reference tissue; data not shown).

Figure 1, *E* and *F* shows that primer p7 (*cf.* Table 1) revealed the presence of CCK_C receptor mRNA in each sample tested, as did primer p8 (Tables 1 and 2). These data suggest that this third type of receptor, with its ability to bind gastrin, might be responsible for part of the gastrin-mediated biologic effects on human melanoma cells, as described in detail below. There are no commercially available antibodies that reveal the CCK_C receptors at protein level.

Characterization of the Influence of Gastrin on Overall Growth Rates, Cell Cycle Kinetics, Motility Levels, and the Organization of Actin Cytoskeleton in the Four Human Melanoma Cell Lines under Study

In vitro, gastrin did not significantly modify overall growth rates (for concentrations ranging from 10^{-12} to 10^{-8} M) in the four human melanoma cell lines under study (data not shown). Gastrin did not therefore significantly modify cell cycle kinetics (i.e., the percentages of tumor cells in the G1, S, and G2/M phases of their cell cycles) in the cell lines under study (data not shown).

Cell migration is a very complex biologic process [29,30] that incorporates cell adhesion [interactions between the cells and the extracellular matrix (ECM)] [31,32], cell motility (cell movement that is dependent on the organization of the actin cytoskeleton in relation to the attachment to the ECM components) [33,34], and invasion (the process of biologic degradation, as illustrated in the "Characterization of the Influence of Gastrin on the Invasiveness Levels of Human Melanoma Cells and Expression of MMP-14"). Figure 2*A* shows that gastrin significantly modifies motility levels in HT-144 and C32 human melanoma cells, but not in G-361 and SKMEL-28 cells.

Integrins, which are dimeric transmembrane glycoproteins linking the intracytoplasmic actin cytoskeleton to the ECM components during cell migration [29–34], also play various major roles in melanoma cell migration [35,36]. Figure 2*B* illustrates the pattern of integrin mRNA expression in human

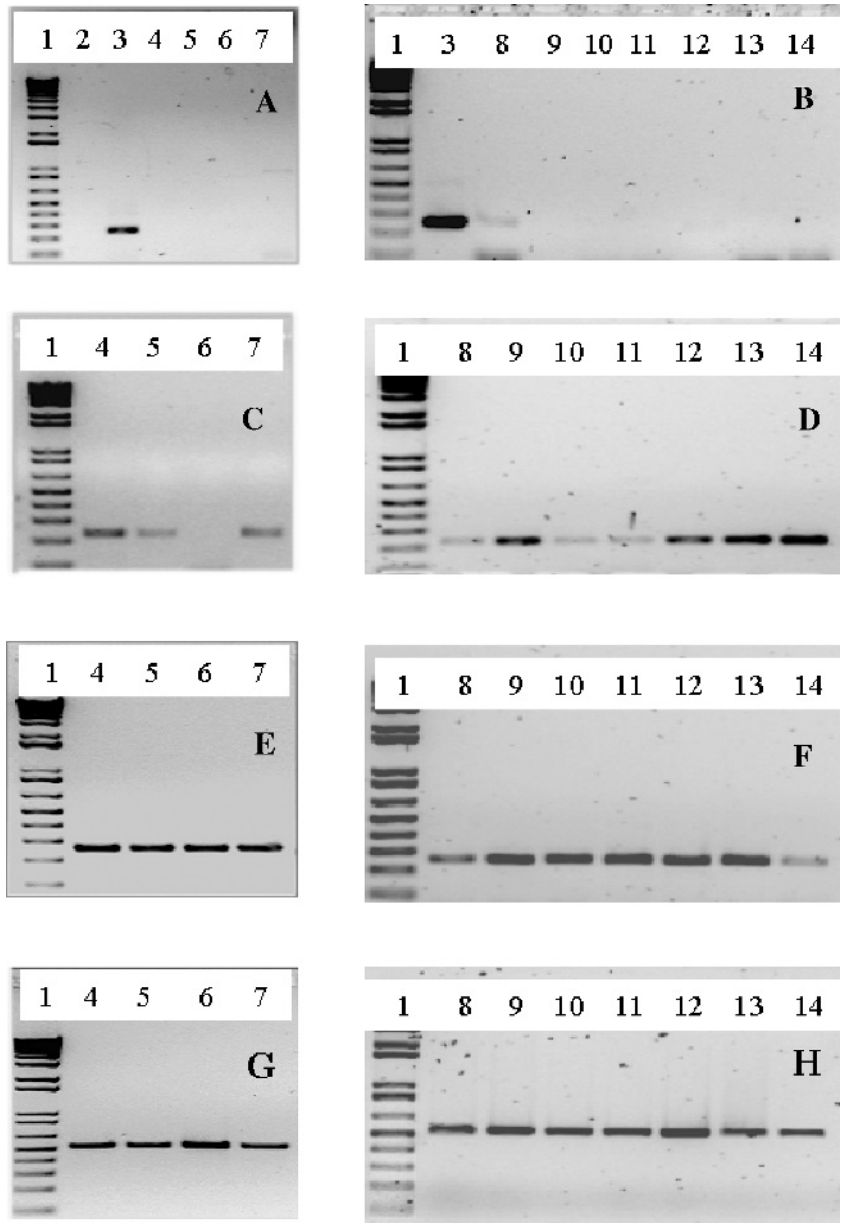

Figure 1. *Illustration of the mRNA expression pattern of the CCK_A, CCK_B, and CCK_C receptors in four human melanoma cell lines (lanes 4–7 in A, C, and E) and seven human melanoma metastases (lanes 8–14 in B, D, and F) assessed by means of RT-PCR. Lane 1: kilobase plus ladder; lane 2: negative control; lane 3: gallbladder tissue (positive control for the CCK_A receptor); lane 4: HT-144 cell line; lane 5: G-361 cell line; lane 6: C32 cell line; lane 7: SKMEL-28 cell line. (A and B) The results obtained with primer p2, which we used for the CCK_A receptor (cf. Table 1). (C and D) Those obtained with primer p6, which we used for the CCK_B receptor (cf. Table 1). (E and F) Those obtained with primer p7, which we used for the CCK_C receptor (cf. Table 1). An RT-PCR for β-actin was performed with all the extracts as quality control (G and H).*

HT-144 cells on which gastrin exerts a potent antimotility effect (Figure 2*A*). $\alpha_v\beta_3$ integrin plays various important roles during the transition of melanoma cells from the radial to the vertical growth phase [35,36]. Figure 2*B* shows that $\alpha_v\beta_3$ integrin was well expressed in the HT-144 cells, at least at the mRNA level, as was also the case for the other three human melanoma cell lines under study (data not shown). Figure 2*C* shows that, in HT-144 cells, 10 nM gastrin significantly decreased the levels of expression (at protein level) of both α_v and β_3 integrin subunits. The gastrin-induced decrease in the expression of the α_v and β_3 integrins in the HT-144 cells (Figure 2*C*) could at least partly explain the gastrin-induced decrease in the motility levels of these cells (Figure 2*A*). In fact, minor modifications to the forces generated by the adhesion of specific types of integrins to various components of the ECM can markedly modify cell migration rates [37].

As indicated above, the organization of the actin cytoskeleton is crucial for cell migration, and gastrin is able to modulate the organization of the actin cytoskeleton through multiple pathways [7,21]. Figure 2, *D* and *E* illustrates the actin cytoskeleton pattern in the untreated HT-144 cells (Figure 2*D*) and in the HT-144 cells treated with 10 nM gastrin

Table 2. Rundown of the Data on the Various Primers Set Up to Evidence the Presence of CCK_A, CCK_B, and CCK_C Receptor mRNA in the Four Human Melanoma Cell Lines and in the Seven Clinical Samples (the Primers Used are Described in Table 1).

Receptors	CCK_A-R				CCK_B receptor		CCK_C receptor	
Primers	p1	p2	p3	p4	p5	p6	p7	p8
Cell lines								
HT-1444	–	–	–	+	–	+	+	+
G-361	–	–	–	–	–	+	+	+
C32	–	–	–	+	–	–	+	+
SKMEL-28	–	–	+	+	–	+	+	+
Clinical samples								
CS-1	–	–	+	+	–	+	+	+
CS-2	–	–	–	+	–	+	+	+
CS-3	–	–	–	+	–	+	+	+
CS-4	–	–	–	–	–	+	+	+
CS-5	–	–	–	+	–	+	+	+
CS-6	–	–	–	+	–	+	+	+
CS-7	–	–	+	+	+	+	+	+

The exact locations in the mRNA of the three receptors targeted by the various primers are illustrated in Table 1.

for 3 hours (Figure 2*E*). Figure 2*F* illustrates the log fibrillar (F) actin/globular (G) actin ratio in the untreated (Ct condition) HT-144 cells or in the cells treated with 10 nM gastrin for 3 and 12 hours; the data show that gastrin markedly increased the proportion of fibrillar actin to the detriment of globular actin—a factor that could rigidify the actin cytoskeleton and may, in turn, decrease the suppleness required for optimal migration (*cf.* Figure 2*A*).

Characterization of the Influence of Gastrin on the Invasiveness Levels of Human Melanoma Cells and Expression of MMP-14

Gastrin significantly decreased the invasiveness levels of the HT-144 (Figure 3*A*), G-361 (Figure 3*B*), and SKMEL-28 (data not shown) human melanoma cells in a dose-dependent manner. This was not, however, the case for the C32 cells, which exhibited no invasive abilities in the context of the Matrigel-coated Boyden chambers (data not shown).

We wished to ascertain whether gastrin was able to modify the levels of expression of one or other MMPs because MMPs play various crucial roles in invasion processes. Figure 3*C* shows that of the MMPs expressed by the HT-144 cells, MMP-14, MMP-15, MMP-16, and MMP-20 were strongly expressed, at least at mRNA level, as were TIMP-1, TIMP-2, and TIMP-3. Similar patterns of expression were observed for the remaining three cell lines under study (data not shown). As described in detail in the "Discussion," there are several studies that clearly indicate the involvement of MMP-14 in the biologic aggressiveness of melanomas [38–41]. We therefore investigated whether gastrin is able to modify the levels of expression of MMP-14 in HT-144 cells. Figure 3*D* clearly indicates that 10 nM gastrin (but not 0.1 nM) significantly decreased the levels of expression of MMP-14

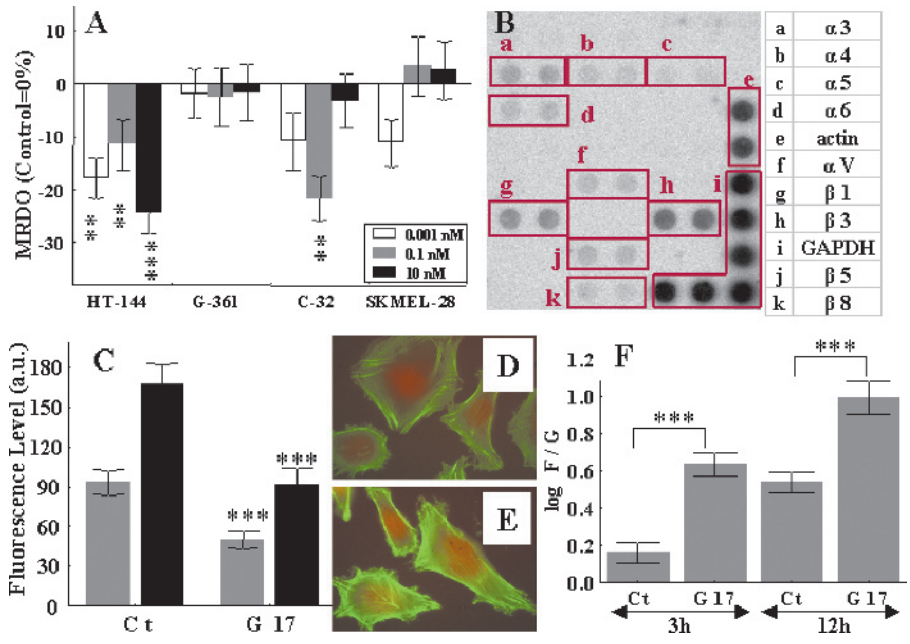

Figure 2. *(A) Determination of the influence of gastrin on motility levels (quantified by means of computer-assisted phase-contrast microscopy) in the HT-144, G-361, C32, and SKMEL-28 human melanoma cells. The motility levels are related to the maximum relative distance from the point of origin (in μm/hr) traveled by each cell analyzed individually over an 8-hour period of observation. The control value was arbitrarily normalized to "0%." (B) Human HT-144 melanoma cells were subjected to a cDNA microarray to determine the pattern of integrin-specific RNA expression. (C) Characterization of the influence of 10 nM gastrin (G17) on the levels of expression of α_v (gray bars in C) and β_3 (black bars in C) integrin subunits in HT-144 human melanoma cells 24 hours after the addition of gastrin to the melanoma cell culture medium. The levels of integrin subunit expression were determined by means of computer-assisted fluorescence microscopy. (D and E) The morphologic appearance of fibrillar (green fluorescence) as opposed to globular (red fluorescence) actin in the cytoskeletons of the untreated HT-144 cells (D) and those treated for 3 hours with 10 nM G17 (E). The ratio of fibrillar/globular actin was quantitatively determined by means of computer-assisted fluorescence microscopy (F). The data are presented as means (thick bars) ± standard error of the mean (thin bars). **P < .01 and ***P < .001 in comparison with the control.*

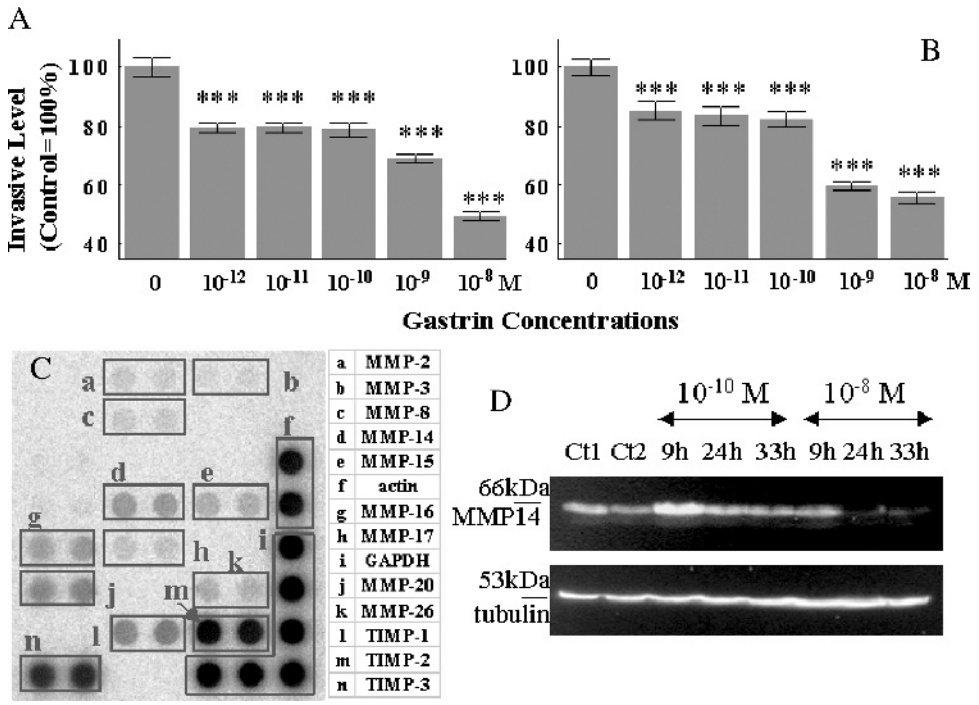

Figure 3. *Characterization of the influence of gastrin (from 10^{-12} to 10^{-8} M) on the invasiveness (in Boyden chambers coated with Matrigel) of HT-144 (A) and G-361 (B) human melanoma cells. (C) HT-144 human melanoma cells were subjected to a cDNA microarray to determine the pattern of MMP-specific RNA expression. (D) The gastrin-induced effects on the MMP-14 expression. Western blot analysis was performed 9, 24, and 33 hours after the addition of 0.1 and 10 nM gastrin (except in the control, Ct) to the culture medium of human HT-144 melanoma cells.*

for between 9 and 33 hours after its addition to the HT-144 cell culture medium.

Characterization of the Influence of a Combination of Gastrin and a Cytotoxic Agent (Cisplatin or Dacarbazine) on the In Vitro *Development of Human HT-144 Melanoma Cell Populations*

The data reported in the previous sections clearly indicate that gastrin has some marked antimigratory effects on human HT-144 melanoma cells, without affecting their proliferation, however. We therefore investigated what occurs in the development of human HT-144 melanoma cell populations (taking cell proliferation, death, and migration into consideration) when gastrin is combined with a cytotoxic agent. We used the scratch wound assay that enables cell migration, proliferation, and death to be analyzed together. Figure 4*A* shows the result of a scrapping procedure on a subconfluent HT-144 melanoma cell population and the results (Figure 4*B*) of a successful colonization (“wound healing”) of the “mechanical wounds” by migrating and proliferating HT-144 cells. Figure 4*C* illustrates the effects of gastrin pretreatment, which precedes cytotoxic treatment on the wound healing phenomenon. The wound healing surface filled by HT-144 cells cultured in the absence of gastrin and cisplatin was arbitrarily normalized to 100% of wound colonization. The duration of gastrin pretreatments at 10 nM is represented on the *x*-axis, with “0” corresponding to the absence of gastrin pretreatment. In this last condition, cisplatin used at 0.1 μM (*black squares*) or 10 μM (*black dots*) reduced wound healing by 4% and 12%, respectively (compared to the arbitrary 100% control value), a result that did not attain a statistical level of significance ($P > .05$) compared to the control. In sharp contrast, the addition of 10 nM gastrin to the culture medium of the HT-144 cells 3 hours (“−3”), 7 hours (“−7”), or 24 hours (“−24”) prior to the addition of 0.1 or 10 μM cisplatin markedly decreased ($P < .01$) the ability of these HT-144 cells to colonize the wounds, whereas treatment with 10 nM gastrin alone over a 24-hour period did not contribute more than 20% ($P < .05$) of wound healing inhibition (data not shown). A significant additive effect on the abilities of the HT-144 cells to colonize wounds (Figure 4*C*) has already been obtained by treatment with 10 nM gastrin for 1 hour prior to the addition of cisplatin. We obtained identical data when combining dacarbazine with gastrin (data not shown).

Several types of highly malignant and migrating cells, such as glioblastoma cells [31], are protected against apoptosis, and the experimentally induced decrease in migration levels of rapidly migrating cancer cells can restore a certain level of sensitivity of these cancer cells to proapoptotic agents, such as cytotoxic ones [31]. Gastrin has been reported to have antiapoptotic effects on different types of cells [22,23,42]; in this study, we observed that gastrin markedly decreased the levels of migration of HT-144 melanoma cells. As illustrated below, HT-144 cells are resistant to apoptosis. We therefore wished to ascertain whether, in the case of these highly migrating HT-144 melanoma cells, gastrin could restore a certain degree of sensitivity to apoptosis in relation to the gastrin-induced decrease in HT-144 cell migration, or whether

gastrin would continue to have an antiapoptotic effect on the HT-144 cells despite the fact that it is able to significantly decrease the migration rates of these cells.

Figure 4*D* shows that the basal expression of the p53 protein in the HT-144 and C32 melanoma cells is low (or even absent) and that a cytotoxic insult with adriamycin (10 μM) increases its expression, suggesting the potential presence of functional p53 protein in these melanoma cells. In contrast, HT-144 and C32 melanoma cells did not express any *PTEN* gene product, a factor that would render them resistant to apoptosis (Figure 4*D*). The data illustrated in Figure 4*E* fully support this hypothesis. In fact, the open bars in Figure 4*E*

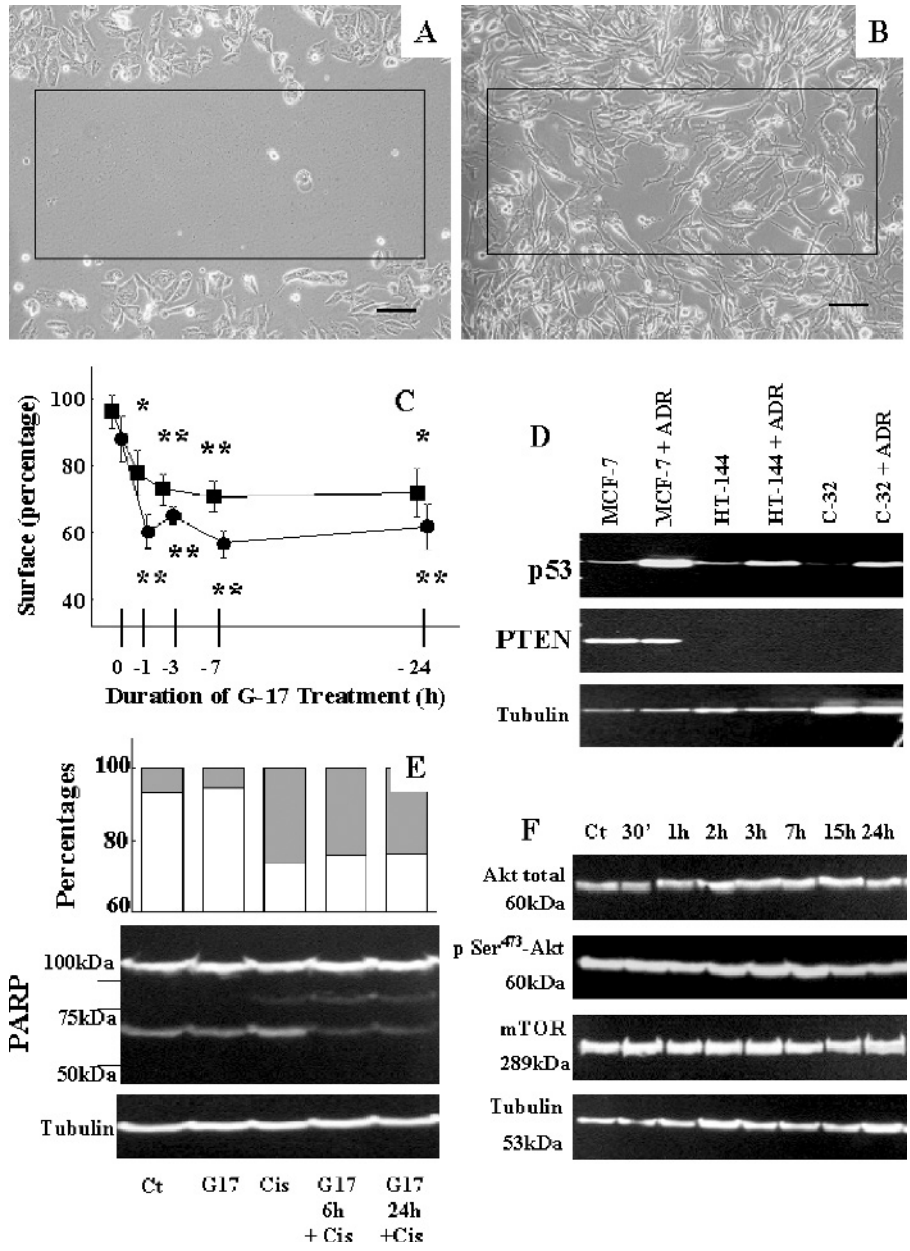

Figure 4. *Therapeutic combination of gastrin and cytotoxic agents in scratch wound assay. Colonization of a mechanical wound in a subconfluent HT-144 cell population (cf. black rectangles in A) was followed over time (complete wound healing is shown in B). (C) The delay in the wound healing process obtained when HT-144 cells were submitted to gastrin treatment [10 nM; duration of the gastrin exposure varied from 0 hour (no gastrin treatment) to 24 hours on the x-axis] before the cytotoxic insult with cisplatin used at 0.1 μM (black squares) or 1 μM (black dots). Control took the shape of HT-144 cells cultured in the absence of gastrin and cisplatin and was arbitrarily normalized to 100% of wound colonization after 62 hours of culture. (D) Western blot analyses of the levels of expression of p53 and PTEN in human MCF-7 breast cancer (positive control) and human melanoma HT-144 and C32 melanoma cells either challenged with 10 μM adriamycin (ADR) or left unchallenged. Cell death processes were investigated by means of flow cytometry and Western blot analysis for PARP, as shown in (E). The open bars in the upper part represent normal cells (i.e., cells not in the process of dying), the black bars (located between the open and gray bars, and so thin that they are almost invisible) represent the proportion of apoptotic cells, and the gray bars represent the proportion of cells dying from nonapoptotic processes. The bottom part of (E) deals with the Western blot analysis. The experimental conditions on the x-axis are as follows: Ct = control; G17 = 10 nM gastrin for 24 hours; G17 6 hours + Cis = 10 nM gastrin added 6 hours before 10 μM cisplatin, with PARP cleavage determination carried out 48 hours after the addition of cisplatin to the culture medium of HT-144 cells; G17 24 hours + Cis = 10 nM gastrin 24 hours before 10 μM cisplatin, with PARP cleavage determination carried out 48 hours after the addition of cisplatin to the culture medium of the HT-144 cells. (F) Characterization over time of the effects of 10 nM gastrin on the levels of expression and activation (^{473}Ser-phospho-Akt) of Akt and on the levels of expression of mTOR.*

represent normal cells (i.e., cells that are not dying), the black bars (located between the open and gray bars, and so thin that they are almost invisible) represent the ratio of apoptotic cells, and the gray bars indicate the ratio of cells dying from nonapoptotic processes. These data show that death in HT-144 melanoma cells undergoing cisplatin or gastrin + cisplatin treatment is nonapoptotic cell death because apoptosis is not present in these cells (Figure 4*E*). Gastrin did not induce any cell death at a 10-nM dose when compared to the control cells, nor did it modify the pattern of HT-144 cell death when compared to cisplatin alone (Figure 4*E*). We performed Western blot analyses of PARP [43,44] to confirm the absence of apoptosis in HT-144 cells. PARP is a 116-kDa enzyme that regulates chromatin structure during differentiation and DNA repair and is prone to proteolytic cleavage, either yielding a 85- to 89-kDa fragment during apoptosis or resulting in 50- to 62-kDa fragments during necrosis [43,44]. The data in Figure 4*E* clearly indicate that 10 μM cisplatin did not induce evident apoptosis or necrosis in the HT-144 melanoma cells, and that 10 nM gastrin even overcame the weak effects induced by 10 μM cisplatin.

Resistance to cell death in many types of cancers is partly related to constitutive activation of the interlinked phosphoinositide 3-kinase (PI3-K) [31,45], Akt [31,46,47], mTOR [31], and NF-κB [31,47] signaling pathways, and to the fact that the *PTEN* gene is no longer active, as is the case with HT-144 cells (Figure 4*D*). PTEN inactivation in HT-144 cells should therefore result in basal activation of the Akt in these cells. This is the case, as is illustrated in Figure 4*F*. In fact, the basal expression of Akt and mTOR is high in HT-144 cells in which activated forms of ^{473}Ser-phospho-Akt (Figure 4*F*) and ^{308}Thr-phospho-Akt (data not shown) are also present even

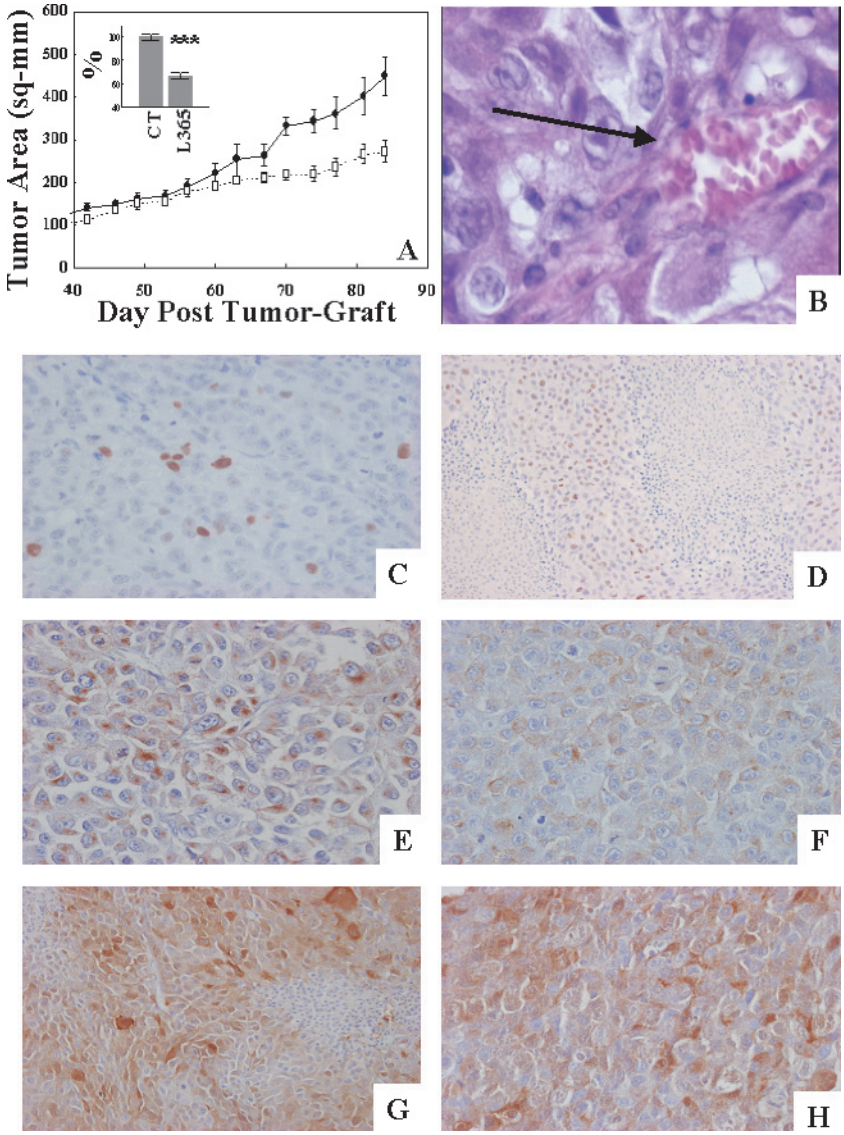

Figure 5. *The effects of the L365,260 CCK_B gastrin receptor antagonist on neoangiogenesis in C32 human melanoma cell xenografts (A). The black dots represent the tumor size of the control group, whereas the white squares represent the one in the group treated with the L365,260 product (10 mg/kg five times a week during an 8-week period). The L365,260-induced decrease in tumor growth can be related to a significant decrease in angiogenesis evidenced by the quantification of the vessels (illustrated in B; cf. black arrow) shown in the upper panel of (A) (Ct: control group; L365: treated group). The expression patterns of p53 and Ki-67 in C32 xenografts are illustrated in (C) (G ×400) and (D) (G ×200), respectively. The immunohistochemical stainings for HMB45 in C32 (E; G ×400) and HT-144 (F; G ×400) tumors as well as for S100B (G; G ×200) (H; G ×400) confirmed the melanoma origin and the melanoma biologic features of these xenografted models.*

though the cells have not been submitted to any cytotoxic insults (i.e., in control, Ct). All these data thus show that human HT-144 cancer cells are protected against apoptosis by defects in the expression of the *PTEN* gene product, with the concomitant activation of Akt (and also the basal activation of mTOR). All these data together thus show that the gastrin-induced decrease in melanoma cell migration did not restore a significant level of sensitivity to apoptosis in these migratory-restricted melanoma cells. It is, however, undeniable that the antimigratory effects contributed by gastrin had a significant additional negative effect on the cytotoxic effects of cisplatin or dacarbazine on the *in vitro* development of HT-144 melanoma cells, when compared to the negative effects of cytotoxic drugs alone on such development.

Characterization of the Influence of a CCK_B Gastrin Receptor Antagonist on In Vivo *Neoangiogenesis in Human C32 Xenografts*

We were the first to recently evidence that gastrin has potent proangiogenic effects and that the CCK_B gastrin receptor antagonist L365,260 contributes a significant amount of therapeutic benefit in the case of an orthotopic model of a human glioblastoma xenograft [9]. Bearing in mind that neoangiogenesis plays various crucial roles in the development of human melanomas [48,49], we therefore wondered whether this finding could be extended to human melanoma xenografts. Figure 5*A* shows that chronic treatment with L365,260 (40 intraperitoneal administrations of 10 mg/kg over a 2-month period of treatment; $P < .01$) decreased the growth of the C32 human xenografts very significantly, knowing that C32 human melanoma cells did not express CCK_B receptors (Figure 1 and Table 2), unlike endothelial cells [9]. We thus counted the vessels, as illustrated in Figure 5*B* (*cf.* black arrow), to assess the levels of neoangiogenesis in the control (Ct), as opposed to the L365,260 (L365)–treated C32 xenografts. The upper panel in Figure 5*A* shows that chronic L365,260-related treatment ($P < .001$) decreased the neoangiogenesis levels in the C32 human melanoma xenografts very significantly—a feature to be analyzed concomitantly with the L365,260-induced decrease in C32 xenograft growth (Figure 5*A*).

The L365,260 treatment induced no statistically significant ($P > .05$) modifications to the percentages of p53-immunopositive cells in the C32 melanoma xenografts, when compared to the control cells (data not shown); the p53 pattern of immunostaining in a C32 xenograft is morphologically illustrated in Figure 5*C*. This suggests that the L365,260 treatment did not modify the C32 tumor cell heterogeneity pattern (i.e., it did not act specifically on any cell subpopulation present in the C32 xenografts). The L365,260 treatment also failed to significantly modify cell proliferation levels in the C32 xenografts (data not shown), as assessed by MIB-1 immunohistochemical analyses (targeting the Ki-67 cell proliferation–related antigen), which is morphologically illustrated in Figure 5*D*.

Figure 5, *E*–*H* clearly indicates that the HT-144 and C32 human melanoma models retained biologic properties characteristic of human melanomas. In fact, both the C32 (Figure 5*E*) and HT-144 (Figure 5*F*) *in vivo* xenografts exhibited a significant immunohistochemical expression of the HMB45 antigen, as was also the case for S100B [Figure 5, *G* (C32 xenograft) and *H* (HT-144 xenograft)].

Discussion

As emphasized by Satyamoorthy et al. [4,5] and Lazar-Molnar et al. [49], cell proliferation, differentiation, adhesion, migration, invasion, apoptosis, stromal formation, and angiogenesis in melanomas are modulated by crosstalks between melanoma cells and their microenvironment through growth factors and cytokines. These authors [4,5,49] report that the basic fibroblast growth factor IGF-1, the platelet-derived growth factors A and B, the transforming growth factors α and β, IL-8, the vascular endothelial growth factor, and the hepatocyte growth/scatter factor are to be found primarily among these factors and cytokines. This study strongly suggests that gastrin can be added to the list of biologic factors that modify melanoma cell biology. In fact, although human melanoma cells seem not to express conventional gastrin receptors (i.e., CCK_A and CCK_B receptors; *cf.* Table 2), gastrin nevertheless markedly modifies the migration factors in various human melanoma cell lines, as is also the case in human glioblastoma cells [12,18,20,21] in which CCK_A and CCK_B receptors are absent [6,12,19,21]. Unlike in human glioblastoma cells where gastrin modifies cell proliferation [17–19], gastrin does not seem to induce any modifications to the growth rates of human melanoma cell lines. Gastrin-induced modifications to human melanoma cell migration could be mediated by the CCK_C receptor (*cf.* Table 2).

Gastrin-induced decrease in human melanoma motility levels might have been partly related to the fact that gastrin decreased the levels of expression of both the α_v and β_3 integrins, which are known to play various important roles in melanoma cell migration [50,51], and to the fact that gastrin modified the organization of the actin cytoskeleton in these human melanoma cells. Sturm et al. [51] report that the expression of the β_3 integrin subunit in melanomas *in situ* has been found to correlate with tumor thickness, ability to invade and metastasize, and poor prognosis.

We had already evidenced gastrin-induced modifications to the organization of the actin cytoskeleton of human melanoma cells in human glioma cells [21]. The fact that gastrin is capable of significantly modifying the organization of the actin cytoskeleton is actually a familiar phenomenon that occurs through gastrin-induced activation of PI3-K–, protein kinase C–, and small GTPase–related pathways [7]. In fact, gastrin-induced modifications to the actin cytoskeleton include gastrin-induced effects on the p60Src/p125FAK complex, which acts upstream of the gastrin-stimulated PI-3K kinase pathway [52]. In this study, we observed that gastrin significantly increased the proportion of stress fibers in the HT-144 cells. Taniguchi et al. [53] not only observed similar features in NIH3T3 fibroblasts into which the CCK_B/gastrin receptor had been introduced through an eukaryotic expression vector, but also the fact that CCK (a gastrin-related peptide) regulated actin stress fiber formation through the CCK_B receptor in a rho p21-dependent manner. The

gastrin-induced effects that we observed at the levels of human melanoma cell motility and actin cytoskeleton organization could not relate to the CCK_B/gastrin receptors because neither this type of receptor nor the CCK_A receptors were present in the human melanoma cells that we studied (*cf.* Table 2). The possibility thus remains that these gastrin-related effects were mediated, at least in part, through the CCK_C receptor or through some GBP, such as the G7 binding site that we recently evidenced in glioma cells [12], or even through an as yet unknown GBP that was more or less specific to melanoma cells.

The gastrin-induced modifications to HT-144 melanoma cell invasiveness through Matrigel seem to be related, at least in part, to gastrin-induced decreases in the levels of expression of MT1–MMP (MMP-14). We decided to concentrate our attention on MMP-14 because it plays various crucial roles in remodeling connective tissue matrices through its ability to initiate zymogen activation cascades, including the activation of pro-MMP-2 and pro-MMP-13 and, indirectly, pro-MMP-9 [41]. Active MT1–MMP is inserted into the plasma membrane with the catalytic domain facing the extracellular space where it can cleave pericellular substrates [41]. The gastrin-induced effects observed here in the case of MMP-14 did not modify the levels of expression and/or activation of MMP-2 and MMP-9 in the HT-144 cells because these two metalloproteinases are expressed weakly, if at all, in HT-144 cells (data not shown). The possibility thus remains that the gastrin-induced decrease in HT-144 cell invasiveness related either to a direct gastrin-induced decrease in the MMP-14 levels of expression or to a gastrin-induced decrease in the MMP-14–mediated activation of the pro-MMP-13, or both. Although the gastrin-mediated regulation of MMP expression has already been demonstrated in human AGS gastric cancer cells in the case of MMP-9 [54], a gastrin-mediated regulation of MMP-14 expression, to our knowledge, has not yet been reported. Gastrin stimulates PI3-K activity [7]. PI3-K is an important regulator of vasculogenic mimicry in aggressive melanoma cells and specifically affects MT1–MMP and MMP-2 activities [40]. The possibility therefore remains that part of the gastrin-mediated effect on MT1–MMP expression in HT-144 melanoma cells (and, subsequently, on their invasiveness through Matrigel) could be mediated by PI3-K.

Our data on MMP-14 can also be analyzed concomitantly with those reported by Naglich et al. [38], who used murine B16BL6 melanoma cells injected into the bloodstream of mice to induce experimental lung metastases. This mouse melanoma model expressed MMP-2 and MMP-14; treatment with BMS-275291, a sulfhydryl-based MMP inhibitor of mice bearing B16BL6 melanoma-related lung metastases, resulted in a significant decrease in MMP-2 and MMP-14 levels of expression and, consequently, in significantly decreased metastasis formation [38].

It is interesting to note that MMP-14 is required at the migration front of melanoma cells [55] and that it has been shown to colocate with integrin $\alpha_v\beta_3$ in melanoma cells [56]. Both these proteins play various major roles in melanoma cell migration, and our study strongly suggests that they are both targeted by gastrin.

Migrating cancer cells can be protected against apoptosis or, more precisely, anoikis, a specific form of apoptosis that occurs when adherent cells detach themselves from their substrates. This is well evidenced in glioblastomas [31], a tumor type in which gastrin plays various major biologic roles [9,17–21]. Decreasing the levels of migrating cancer cells can restore a certain level of sensitivity to apoptosis, and hence to the cytotoxic insults induced by proapoptotic agents [31]. We therefore wondered whether the gastrin-induced decrease in HT-144 cell migration could induce an increased degree of sensitivity of these cells to the pro-apoptotic insults induced by cytotoxic agents, such as cisplatin and dacarbazine. In fact, although gastrin significantly decreased the levels of migration of the HT-144 cells, it did not increase HT-144 cell sensitivity to apoptosis, a feature that can at least partly be explained by two different facts, namely that: 1) HT-144 cells are naturally protected against apoptosis, at least with respect to the constitutive activation of Akt and mTOR (whereas p53 is still inducible in these HT-144 cells; *cf.* Figure 4*D*), and 2) gastrin has potent antiapoptotic effects [22,23,42]. The fact that cisplatin induced cytotoxic effects in these HT-144 cells that appeared to be protected against apoptosis can, at least in part, be explained by cisplatin-induced cell death unrelated to apoptosis, as already demonstrated in the case of several other cells types [57,58]. However, this study clearly demonstrates that adding gastrin to cisplatin or dacarbazine *in vitro* in HT-144 cell population induced a greater delay in the development of the HT-144 tumor cell population in the scratch wound assay than was the case with gastrin or the cytotoxic agent alone. This relates to the combination of both the anti-migratory effects of gastrin and the cytotoxic effects of cisplatin or dacarbazine.

We recently showed that gastrin induces potent proangiogenic effects both *in vitro* in human umbilical vein endothelial cells and *in vivo* in experimental glioblastomas [9]. These gastrin-induced effects occurred, at least in part, through modifications to the expression of E-selectin and P-selectin at the surface of endothelial cells, as well as through associations with chemokines, including IL-8 at least [9]. We therefore wondered whether gastrin could also modify neoangiogenesis levels in experimental *in vivo* human melanoma xenografts. The data in Figure 5 clearly evidence that the chronic use of a selective antagonist of the CCK_B gastrin receptor (that prevents the biologic effects induced by endogenous gastrin) on human C32 melanoma xenografts significantly decreased the growth levels of these C32 tumors with a concomitant decrease in neoangiogenesis levels. These data therefore emphasize the proangiogenic effects for gastrin in experimental melanomas, as already evidenced in experimental glioblastomas [9] and pancreatic cancers (data not shown).

In conclusion, although human melanoma cells seem to be devoid of conventional CCK_A and CCK_B/gastrin receptors, *in vitro* gastrin nevertheless significantly modifies the migration rates of human melanoma cells at both motility and invasion levels. These gastrin-mediated modifications observed in the case of melanoma cell migration could be

mediated by the CCK_C receptor. In contrast, gastrin seems not to induce any modifications to the growth characteristics of human melanoma cells.

References

[1] Lawson DH (2004). Update on the systemic treatment of malignant melanoma. *Semin Oncol* **31,** 33–37.

[2] Kim KB, Eton O, East MJ, Hodges C, Papadopoulos NE, Grimm EA, and Bedikian AY (2004). Pilot study of high-dose, concurrent biochemotherapy for advanced melanoma. *Cancer* **101,** 596–603.

[3] Pollak MN, Schernhammer ES, and Hankinson SE (2004). Insulin-like growth factors and neoplasia. *Nat Rev Cancer* **4,** 505–518.

[4] Satyamoorthy K, Li G, Vaidya B, Patel D, and Herlyn M (2001). Insulin-like growth factor-1 induces survival and growth of biologically early melanoma cells through both the mitogen-activated protein kinase and β-catenin pathways. *Cancer Res* **61,** 7318–7324.

[5] Satyamoorthy K, Li G, Vaidya B, Kalabis J, and Herlyn M (2002). Insulin-like growth factor-1–induced migration of melanoma cells is mediated by interleukin-8 induction. *Cell Growth Differ* **13,** 87–93.

[6] Reubi JC, Schaer JC, and Waser B (1997). Cholecystokinin (CCK)-A and CCK-B/gastrin receptors in human tumors. *Cancer Res* **57,** 1377–1386.

[7] Rozengurt E and Walsh JH (2001). Gastrin, CCK, signaling, and cancer. *Annu Rev Physiol* **63,** 49–76.

[8] Dockray GJ, Varro A, Dimaline R, and Wang T (2001). The gastrins: their production and biological activities. *Annu Rev Physiol* **63,** 119–139.

[9] Lefranc F, Mijatovic T, Mathieu V, Rorive S, Decaestecker C, Debeir O, Brotchi J, Van Ham P, Salmon I, and Kiss R (2004). Characterization of gastrin-induced proangiogenic effects *in vivo* in orthotopic U373 experimental human glioblastomas, and *in vitro* in human umbilical vein endothelial cells (HUVEC). *Clin Cancer Res* **10,** 8250–8265.

[10] Rehfeld JF and Van Solinge WW (1994). The tumor biology of gastrin and cholecystokinin. *Adv Cancer Res* **63,** 295–347.

[11] Baldwin GS, Chandler R, Scanlon DB, and Weinstock J (1986). Identification of a gastrin binding protein in porcine gastric mucosal membranes by covalent cross-linking with iodinated gastrin. *J Biol Chem* **261,** 12252–12257.

[12] Pannequin J, Oiry C, Morel C, Kucharczak J, Camby I, Kiss R, Gagne D, Galleyrand JC, and Martinez J (2002). C-terminal heptapeptide of gastrin inhibits astrocytomas cell motility by interacting with a new gastrin-binding site. *J Pharmacol Exp Ther* **302,** 274–282.

[13] Smith JP, Verderame MF, McLaughlin P, Martenis M, Ballard E, and Zagon IS (2002). Characterization of the CCK-C (cancer) receptor in human pancreatic cancer. *Int J Mol Med* **10,** 689–694.

[14] Seva C, Dickinson CJ, and Yamada T (1994). Growth promoting effects of glycine-extended progastrin. *Science* **265,** 410–412.

[15] Singh P, Owlia A, Espeijo R, and Dai B (1995). Novel gastrin receptors mediate mitogenic effects of gastrin and processing intermediates of gastrin on Swiss 3T3 fibroblasts. Absence of detectable cholecystokinin (CCK)-A and CCK-B receptors. *J Biol Chem* **270,** 8429–8438.

[16] Rehfeld JF, Larsson LI, Goltermann NR, Schwartz TW, Holst JJ, Jensen SL, and Morley JS (1980). Neural regulation of pancreatic hormone secretion by the C-terminal tetrapeptide of CCK. *Nature* **284,** 33–38.

[17] Camby I, Salmon I, Danguy A, Pasteels JL, Brotchi J, Martinez J, and Kiss R (1996). Influence of gastrin on human astrocytic tumor cell proliferation. *J Natl Cancer Inst* **88,** 594–600.

[18] De Hauwer C, Camby I, Darro F, Migeotte I, Decaestecker C, Verbeek C, Danguy A, Pasteels JL, Brotchi J, Salmon I, et al. (1998). Gastrin inhibits motility, decreases cell death levels and increases proliferation in human glioblastoma cell lines. *J Neurobiol* **37,** 373–382.

[19] Lefranc F, Sadeghi N, Metens T, Brotchi J, Salmon I, and Kiss R (2003). Characterization of gastrin-induced cytostatic effect on cell proliferation in experimental gliomas. *Neurosurgery* **52,** 881–891.

[20] Kucharczak J, Pannequin J, Camby I, Decaestecker C, Kiss R, and Martinez J (2001). Gastrin induces over-expression of genes involved in human U373 glioblastoma cell migration. *Oncogene* **20,** 7021–7028.

[21] Lefranc F, Camby I, Belot N, Bruyneel E, Chaboteaux C, Brotchi J, Mareel M, Salmon I, and Kiss R (2002). Gastrin significantly modifies the migratory abilities of experimental glioma cells. *Lab Invest* **82,** 1241–1252.

[22] Stepan V, Ramamoorthy S, Pausawasdi N, Logsdon CD, Askari FK, and Todisco A (2004). Role of small GTP binding proteins in the growth-promoting and antiapoptotic actions of gastrin. *Am J Physiol Gastrointest Liver Physiol* **287,** G715–G725.

[23] Harris JC, Clarke PA, Awan A, Jankowski J, and Watson SA (2004). An antiapoptotic role for gastrin and the gastrin/CCK-2 receptor in Barrett's esophagus. *Cancer Res* **64,** 1915–1919.

[24] Belot N, Rorive S, Doyen I, Lefranc F, Bruyneel E, Dedecker R, Micik S, Brotchi J, Decaestecker C, Salmon I, et al. (2001). Molecular characterization of cell substratum attachments in human glial tumors relates to prognostic features. *Glia* **36,** 375–390.

[25] Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, and Traganos F (1997). Cytometry in cell neurobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* **27,** 1–20.

[26] de Launoit Y, Veilleux R, Dufour M, Simard J, and Labrie F (1991). Characteristics of the biphasic action of androgens and of the potent antiproliferative effects of the new pure antiestrogen EM-139 on cell cycle kinetic parameters in LNCaP human prostatic cancer cells. *Cancer Res* **51,** 5165–5170.

[27] Hittelet A, Camby I, Nagy N, Legendre H, Bronckart Y, Decaestecker C, Kaltner H, Nifant'ev NE, Bovin NV, Pector JC, et al. (2003). Binding sites for Lewis antigens are expressed by human colon cancer cells and negatively affect their migration. *Lab Invest* **83,** 777–787.

[28] Camby I, Nagy N, Lopes MB, Schafer BW, Maurage CA, Ruchoux MM, Murmann P, Pochet R, Heizmann CW, Brotchi J, et al. (1999). Supratentorial pilocytic astrocytomas, astrocytomas, anaplastic astrocytomas and glioblastomas are characterized by a differential expression of S100 proteins. *Brain Pathol* **9,** 1–19.

[29] Lauffenburger DA and Horwitz AF (1996). Cell migration: a physically integrated molecular process. *Cell* **84,** 359–369.

[30] Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, and Horwitz AR (2003). Cell migration: integrating signals from front to back. *Science* **302,** 1704–1709.

[31] Lefranc F, Brotchi J, and Kiss R (2005). Possible future issues in the treatment of glioblastomas, with a special emphasis on cell migration and the resistance of migrating glioma cells to apoptosis. *J Clin Oncol* **23,** 2411–2422.

[32] Hood JD and Cheresh DA (2002). Role of integrins in cell invasion and migration. *Nat Rev Cancer* **2,** 91–100.

[33] Fenteany G and Zhu S (2003). Small-molecule inhibitors of actin dynamics and cell motility. *Curr Top Med Chem* **3,** 593–616.

[34] Rao J and Li N (2004). Microfilament actin remodeling as a potential target for cancer drug development. *Curr Cancer Drug Targets* **4,** 345–354.

[35] Seftor RE, Seftor EA, and Hendrix MJ (1999). Molecular role(s) for integrins in human melanoma invasion. *Cancer Metastasis Rev* **18,** 359–375.

[36] McGary EC, Lev DC, and Bar-Eli M (2002). Cellular adhesion pathways and metastatic potential of human melanoma. *Cancer Biol Ther* **1,** 459–465.

[37] Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, and Horwitz AF (1997). Integrin-ligand binding properties govern cell migration speed through cell–substratum adhesiveness. *Nature* **385,** 537–540.

[38] Naglich JG, Jure-Kunkel M, Gupta E, Fargnoli J, Henderson AJ, Lewin AC, Talbott R, Baxter A, Bird J, Savopoulos R, et al. (2001). Inhibition of angiogenesis and metastasis in two murine models by the matrix metalloproteinase inhibitor, BMS-275291. *Cancer Res* **61,** 8480–8485.

[39] Seftor RE, Seftor EA, Koshikawa N, Meltzer PS, Gardner LM, Bilban M, Stetler-Stevenson WG, Quaranta V, and Hendrix MJ (2001). Cooperative interactions of laminin 5 γ2 chain, matrix metalloproteinase-2, and membrane type-1-matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. *Cancer Res* **61,** 6322–6327.

[40] Hess AR, Seftor EA, Seftor REB, and Hendrix MJC (2003). Phosphoinositide 3-kinase regulates membrane type 1–matrix metalloproteinase (MMP) and MMP-2 activity during melanoma cell vasculogenic mimicry. *Cancer Res* **63,** 4757–4762.

[41] Osenkowski P, Toth M, and Fridman R (2004). Processing, shedding, and endocytosis of membrane type 1–matrix metalloproteinase (MT1–MMP). *J Cell Physiol* **200,** 2–10.

[42] Todisco A, Ramamoorthy S, Witham T, Pausawasdi N, Srinivasan S, Dickinson CJ, Askari FK, and Krametter D (2001). Molecular mechanisms for the anti-apoptotic action of gastrin. *Am J Physiol Gastrointest Liver Physiol* **280,** G298–G307.

[43] Sperandio S, de Belle I, and Bredesen DE (2000). An alternative, non-apoptotic form of programmed cell death. *Proc Natl Acad Sci USA* **97,** 14376–14381.

[44] Casiano CA, Ochs RL, and Tan EM (1998). Distinct cleavage products of nuclear proteins in apoptosis and necrosis revealed by autoantibody probes. *Cell Death Differ* **5,** 183–190.

[45] Lee HY, Srinivas H, Xia D, Lu Y, Superty R, La Pushin R, Gomez-Manzano C, Gal AM, Walsh GL, Force T, et al. (2003). Evidence that

phosphatidylinositol 3-kinase– and mitogen-activated protein kinase kinase-4/c-Jun NH_2-terminal kinase-dependent pathways cooperate to maintain lung cancer cell survival. *J Biol Chem* **278,** 23630–23638.

[46] Castillo SS, Brognard J, Petukhov PA, Zhang C, Tsurutani J, Granville CA, Li M, Jung M, West KA, Gills JG, et al. (2004). Preferential inhibition of Akt and killing of Akt-dependent cancer cells by rationally designed phosphatidylinositol ether lipid analogues. *Cancer Res* **64,** 2782–2792.

[47] Mayo MW, Denlinger CE, Broad RM, Yeung F, Reilly ET, Shi Y, and Jones DR (2003). Ineffectiveness of histone deacetylase inhibitors to induce apoptosis involves the transcriptional activation of NF-kappa B through the Akt pathway. *J Biol Chem* **278,** 18980–18989.

[48] Lu W and Scroit AJ (2005). Vascularization of melanoma by mobilization and remodelling of preexisting latent vessels to patency. *Cancer Res* **65,** 913–918.

[49] Lazar-Molnar E, Hegyesi H, Toth S, and Falus A (2000). Autocrine and paracrine regulation by cytokines and growth factors in melanoma. *Cytokine* **12,** 547–554.

[50] Janji B, Melchior C, Gouon V, Vallar L, and Kieffer N (1999). Autocrine TGF-β–regulated expression of adhesion receptors and integrin-linked kinase in HT-144 melanoma cells correlates with their metastatic phenotype. *Int J Cancer* **83,** 255–262.

[51] Sturm RA, Satyamoorthy K, Meier F, Gardiner BB, Smit DJ, Vaidya B, and Herlyn M (2002). Osteonectin/SPARC induction by ectopic β3 integrin in human radial growth phase primary melanoma cells. *Cancer Res* **62,** 226–232.

[52] Daulhac L, Kowalski-Chauvel A, Pradayrol L, Vaysse N, and Seva C (1999). Gastrin stimulates the formation of a p60Src/p125FAK complex upstream of the phosphatidylinositol 3-kinase signalling pathway. *FEBS Lett* **445,** 251–255.

[53] Taniguchi T, Takaishi K, Murayama T, Ito M, Iwata N, Chihara K, Sasaki T, Takai Y, and Matsui T (1996). Cholecystokinin-B/gastrin receptors mediate rapid formation of actin stress fibers. *Oncogene* **12,** 1357–1360.

[54] Wroblewski LE, Pritchard DM, Carter S, and Varro A (2002). Gastrin-stimulated gastric epithelial cell invasion: the role and mechanism of increased matrix metalloproteinase 9 expression. *Biochem J* **365,** 873–879.

[55] Nakahara H, Howard L, Thompson EW, Sato H, Seiki M, Yeh Y, and Chen WT (1997). Transmembrane/cytoplasmic domain–mediated membrane type 1–matrix metalloprotease docking to invadopodia is required for cell invasion. *Proc Natl Acad Sci USA* **94,** 7959–7964.

[56] Hofmann UB, Westphal JR, Van Kraats AA, Ruiter DJ, and Van Muijen GN (2000). Expression of integrin alpha(v)beta(3) correlates with activation of membrane-type matrix metalloproteinase-1 (MT1–MMP) and matrix metalloproteinase-2 (MMP-2) in human melanoma cells *in vitro* and *in vivo*. *Int J Cancer* **87,** 12–19.

[57] Gonzalez VM, Fuertes MA, Alonso C, and Perez JM (2001). Is cisplatin-induced cell death always produced by apoptosis? *Mol Pharmacol* **59,** 657–663.

[58] Ferrer M, Izeboud T, Ferreira CG, Span SW, Giaccone G, and Kruyt FA (2003). Cisplatin triggers apoptotic or nonapoptotic cell death in Fanconi anemia lymphoblasts in a concentration-dependent manner. *Exp Cell Res* **286,** 381–395.